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Screening of DFNB3 in Iranian families with autosomal recessive non-syndromic hearing loss reveals a novel pathogenic mutation in the MyTh4 domain of the *MYO15A* gene in a linked family

Somayeh Reiisi[1], Mohammad Amin Tabatabaiefar[2], Mohammad Hosein Sanati [3], Morteza Hashemzadeh Chaleshtori[4]*

[1] Department of Genetics, Faculty of Basic Sciences, University of Shahrekord, Shahrekord, Iran
[2] Medical Genetics Dept., Isfahan University of Medical Sciences, Isfahan, Iran
[3] Medical Genetics Dept., National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[4] Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Objective(s): Non-syndromic sensorineural hearing loss (NSHL) is a common disorder affecting approximately 1 in 500 newborns. This type of hearing loss is extremely heterogeneous and includes over 100 loci. Mutations in the *GJB2* gene have been implicated in about half of autosomal recessive non-syndromic hearing loss (ARNSHL) cases, making this the most common cause of ARNSHL. For the latter form of deafness, most frequent genes proposed include *GJB2*, *SLC26A4*, *MYO15A*, *OTOF*, and *CDH23* worldwide.

Materials and Methods: The aim of the present study was to define the role and frequency of *MYO15A* gene mutation in Iranian families. In this study 30 Iranian families were enrolled with over three deaf children and negative for *GJB2*. *Then* linkage analysis was performed by six DFNB3 short tandem repeat markers. Following that, mutation detection accomplished using DNA sequencing.

Results: One family (3.33%) showed linkage to DFNB3 and a novel mutation was identified in the *MYO15A* gene **(**c.6442T>A**)** as the disease-causing mutation. Mutation co-segregated with hearing loss in the family but was not present in the 100 ethnicity-matched controls.

Conclusion: Our results confirmed that the hearing loss of the linked Iranian family was caused by a novel missense mutation in the MYO15A gene. This mutation is the first to be reported in the world and affects the first MyTH4 domain of the protein.

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Introduction

Hearing loss (HL) is the most common sensory disorder estimated to affect 70 million people worldwide. This disorder is mostly a birth defect that affects 1 in 500 newborns (1). HL is extremely heterogeneous and can result from genetic or environmental factors or both (2). More than 50% of hearing loss cases are caused by genetic factors, of which 70% are non-syndromic (NSHL) and the remaining 30% are attributed to syndromic forms (3). Different loci have been identified for NSHL and have been named according to their mode of inheritance (DFNB for autosomal recessive, DFNA for autosomal dominant, and DFN for X-linked) and the time of their identification (ex. DFNB1-DFNB95) (4). Over 60 genes have been discovered causing NSHL (Hereditary hearing loss homepage) (5).Studies have indicated that *GJB2* gene mutations are a major cause of ARNSHL. The contribution of other loci to the ARNSHL is much lower. The extreme heterogeneity of HL makes it cumbersome to identify the genetic cause of this disease in single families leading to difficulties in their genetic counseling and testing. Despite previous intensive GLA and candi-date gene screening, a large proportion of ARNSHL remains genetically unexplained (5, 6). Due to the high heterogeneity of HL, researchers have suggested studying of large families in populations like middle east in which consanguineous marriage rate is high (7). Iran, with specific population characteristics

Corresponding author:Morteza Hashemzadeh Chaleshtori, Cellular and Molecular Research Center, School of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran. Tel: +98 381 3335654, Fax: +98 381 3330709, email: mchalesh@yahoo.com

such as high consanguineousmarri-age rate and heterogeneous population, offers a good opportunity to study rare autosomal recessive disorders including ARNSHL. Studies in Iran showed that contribution proportion of *GJB2* mutations in HL in different populations varies ranging from approximately 27%–38% in the north to 0%–0.4% in the southeast(8-10). So far, the contribution of mutations in other genes associated with HL has been identified only in a limited number of Iranian families and it seems in order to determine a more exact contribution of each one of these genes in Iranian families, more extensive studies are required to be done. For ARNSHL, the most frequent causative genes in order of frequency are *GJB2*, *SLC26A4*, *MYO15A*, *OTOF*, and *CDH23*. Mutations in the SLC26A4 gene, which is located at the DFNB4 locus are the second most frequent cause of ARNSHL and in order of frequency after *GJB2* and *SLC26A4*, mutations in *MYO15A* is the third most frequent cause of ARNSHL in the world (11). Forty-three mutations have been reported in *MYO15A*, most of which have been found using GLA in consanguineous families from specific countries (12-15).

MYO15A encodes an unconventional myosin XVa, which has a role in stereocilia formation (16). Myosins are molecular motor proteins that drive the moment of actin filaments via ATP hydrolysis to facilitate muscle contraction, organelle trafficking, cell movement, cytokinesis, and signal transduction (17). The protein is unique among unconventional myosins in that it includes a long N-terminal domain (coded by exon 2) that is alternatively spliced to generate distinct class 1 and class 2 protein isoforms. The N-terminal domain is required for normal hearing, as premature stop mutations that result in loss of this domain cause DFNB3 hearing loss (18, 19). Myosin XVa also contains domains that are conserved within the myosin protein family, including the motor domain, IQ motifs(calmodulin/-myosin light chain binding), MyTh4 domains (myosin tail like homology region 4), FERM motifs (*4*.1 protein, *E*zrin, *R*adixin, and *M*oesin), SH3 domain (*S*rc homology *3*), and the PDZ ligand domain. Many missense and nonsense mutations in coding exons of the motor domain, FERM, and MyTh4 domain have been identified which cause profound HL in different populations (20-22) .

In this study, we have characterized an ARNSHL family from the west of Iran and identified a novel mutation in the *MYO15A* gene. This novel mutation has been identified in the highly conserved MyTh4 domain in this family.

Materials and Methods

Sampling and genomic DNA extraction

In this descriptive laboratory study, 30 Iranian families with at least 2 affected children were selected, informational questionnaires were filled out by family members, and clinical assessments were performed, also consent was obtained from all family members. This research has been approved by the National Institute of Genetic Engineering and Biotechnology and Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran. All cases of syndromic hearing loss caused by environmental factors such as ototoxic medications, hepatitis, head trauma, or meningitis were excluded. All families were informed and written consent was taken and 5 ml peripheral blood, in tubes containing 0.5 M EDTA from all available members of all families was obtained. Genomic DNA was extracted from blood samples using the phenol-chloroform standard method. DNA quality (purity and DNA concentration) was checked by spectroscopy (UNICO 2100, USA) (23, 24).

GJB2 mutation screening

At least one affected member from every pedigree was selected for detection of *GJB2* mutations and reaction was carried out in accordance with previous protocols (25). PCR product was run in an 8% polyacrylamide gel electrophoresis (PAGE) at 45 mA for 1.5 hr. DNA bands were visualized by silver staining. A single PCR product of 809 bp was obtained and DNA sequencing of the PCR product was carried out in order to detect any change in the gene.

S-LINK analysis, DFNB3 STR marker, and linkage analysis

For S-Link and LOD score calculation, we used the Easy linkage plus (ver. 5.05) genetic software (26). For S-LINK calculation we used FastSlink version 2.51. Two-point and multipoint parametric LOD scores were calculated using Superlink (ver. 1.6) and GeneHunter (ver. 2.91), respectively. For LOD score calculations using these software packages, inheritance pattern of autosomal recessive, complete penetrance, and disease allele frequency of 0.001 were assumed. Haplopainter version 029.5 software package was used for reconstruction of haplotypes (27). Negative subjects for *GJB2* mutations were selected for linkage analysis. For GLA, 6 different STR markers were used. Upon encountering an uninformative marker, further markers were examined. Table 1 summarizes the markers used in the study and their general characteristics. The criteria for selecting these markers are as following: greater heterogeneity values, shorter fragments in amplicon length and lying close to the known locus. STR markers were selected based on their physical distance found at NCBI UniSTS, and Touchdown program was used for markers. Thermal cycling conditions for amplifying markers were in accordance with previous protocols (25).

Table 1. Genetic STR markers used in this study and their characteristics

Heterozygosity	Size (bp)	Reverse primer	Forward primer	STR
0.72	169-185	GGCCACCATAATCATGTCAGACAAT	GGCCACCATAATCATGTCAGACAAT	D17S921
0.70	159-203	GAGAATCACCTGAACCCG	AATTCAAAGGCTAAAAGCAAAC	D17S1843
0.76	119-131	AAGGGCTTGCTTTGAC	ACTATCCGCCCAATACA	D17S953
0.62	177-187	TGCCTAAACTGCTTTCAGGTGAG	TGCACAGGCCAATTCCTTAC	D17S1857
0.71	103-151	TACATTTAATGCAGGATGCC	CTCTTTGTGCTTGGCAGGGT	D17S740
0.81	139-163	ATATTTCAATATTGTAACCAGTCCC	CCAACATCTAGAATTAATCAGAATC	D17S2196

Mutation screening for the MYO15A gene

Sixty-six exons of the MYO15A gene [NM_016239.3] and flanking regions were amplified using designed primers by the Oligo (ver. 7.05) software. Reaction conditions for amplifying exons in 50 µl was as follows: 2 µl MgCl2 (50 mM), 2.5 µl PCR buffer (10X), 0.5 µl of each of forward and reverse primers (50 PM), 0.5 U Taq DNA polymerase (5U/ul), 1 µl dNTP mix (10 mM) and 1 µl genomic DNA (about 100 ng). PCR was done as follows: 95 ˚C for primary denaturation for 5 min; 35 cycles of 94 ˚C denaturation for 1 min, 59 ˚C annealing temperature for 1 min, 72 ˚C extension for 1 min, and a final extension at 72 ˚C for 5 min. In order to detect any variant in the gene sequence, direct sequencing of amplified exons was performed bi-directionally using ABI 3730XL.

Pathogenicity investigation of novel variant

Co-segregation study in family members was accomplished and investigation of conservative amino acid coding of novel variant confirmed the presence of mutation. The absence of new variation in 100 healthy control samples also confirmed the mutation performed by restriction fragment length polymorphism (RFLP) using *Msc1* restriction enzyme. In the current study, we used the following primers: F-5'ATTTATATGGGCAGGGGCAC3' and R-5' AGGCGGCCAGCAGTGGCC 3' for the amplification.

Results

Most of the subjects in this study exhibited severe to profound bilateral sensorineural hearing loss. 70% of families in this study were consanguineous families. According to the information in the pedigree, the type of hearing loss was autosomal recessive non-syndromic.

Mutation 35delG was identified in 5 families out of 30 families by sequencing of the coding region of the *GJB2* gene. These families were homozygous for this mutation and were eliminated from further analysis. The remaining families were further analyzed for the linkage to DFNB1 using 3 informative markers. However, linkage analysis could not find any other family linked to the DFNB1 locus. For linkage analysis to other loci, first S-LINK values were calculated for the remaining families. 14families had S-LINK values ≥3, 5 families were of S-LINK values <2, and the rest of families had S-LINK values 2 to 3. GLA for the DFNB3 locus was carried out by 6 STR markers. By haplotype analysis, out of the 25 remaining families, one family was found to be linked to this locus and this linked family displayed complete pattern of linkage (Figure 1).

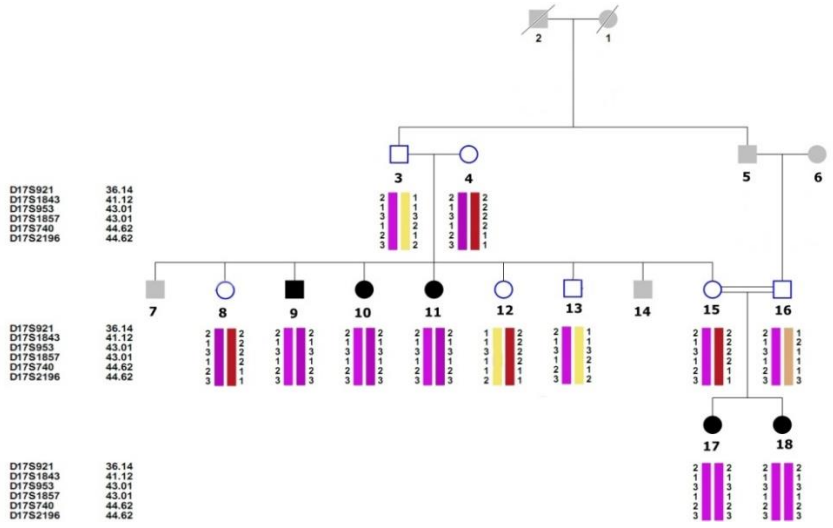

Figure1. Pedigree and haplotypes of the family linked to DFNB3. The order of markers is based on the Marshfield map

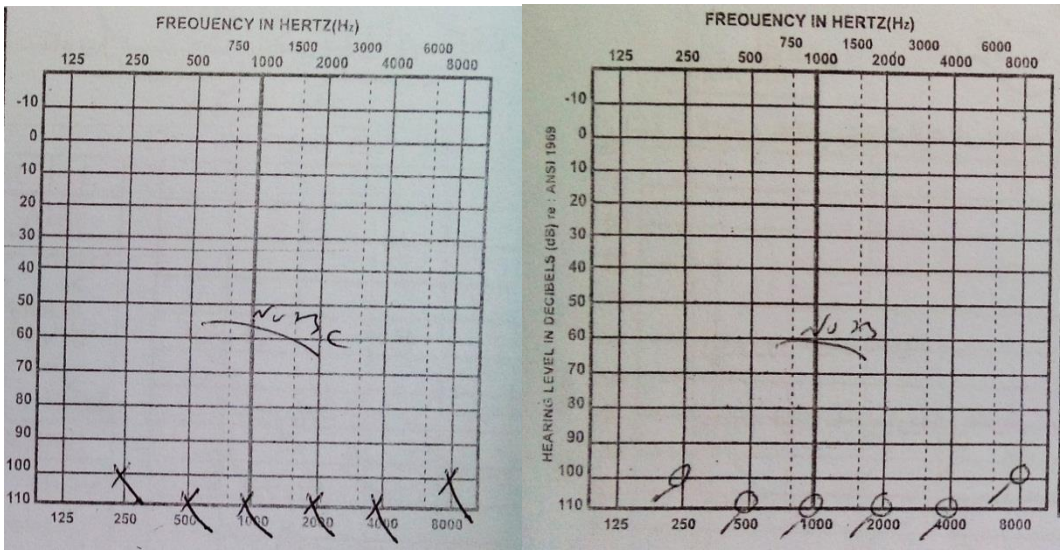

Figure 2. Audiograms for a proband member (18 in Figure 1) of a family linked to DFNB3. "O" indicates air conduction for the right ear, while "X" indicates air conduction for the left ear. Audiograms of the affected individual had shown severe to profound hearing loss at two different time intervals

Audiogram analysis of this family confirmed HL to be profound in all affected subjects of the family (Figure 2). Calculated multipoint LOD score for this family was 3.15, which confirmed the linkage to the locus. DNA sequencing of 66 coding exons of the *MYO15A* gene demonstrated a new variant in the homozygous state in exon 30. We first analyzed all polymorphisms of the gene from all data banks. This novel variant was a missense substitution and caused the substitution of nucleotide T to A in the location 6442 of the coding region of *MYO15A* (c.6442T>A)andsubstitutionoftryptophanfor arginine at residue 2148 (p.Trp2148Arg). The ConSurf web server calculated the conservative score for the residue to be 8, which implies it as highly conserved among diverse species (Figure 3). Co-segregation showed that variant in affected subjects and in parents was in homozygous and heterozygous states, respectively. One normal individual was homozygous for the normal allele(Figure 4). We analyzed the variant in 100 normal controls and no case was found to be positive for it. Thus, the observations would confirm the pathogenicity of the novel variant.

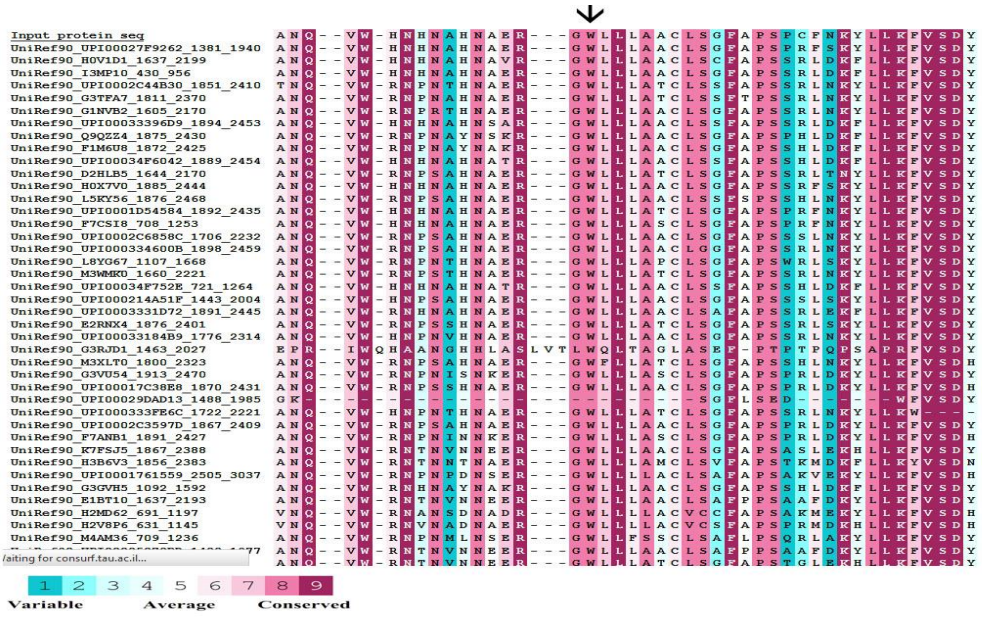

Figure 3.conservity of altered amino acids in different species. Calculated score for amino acid was 8

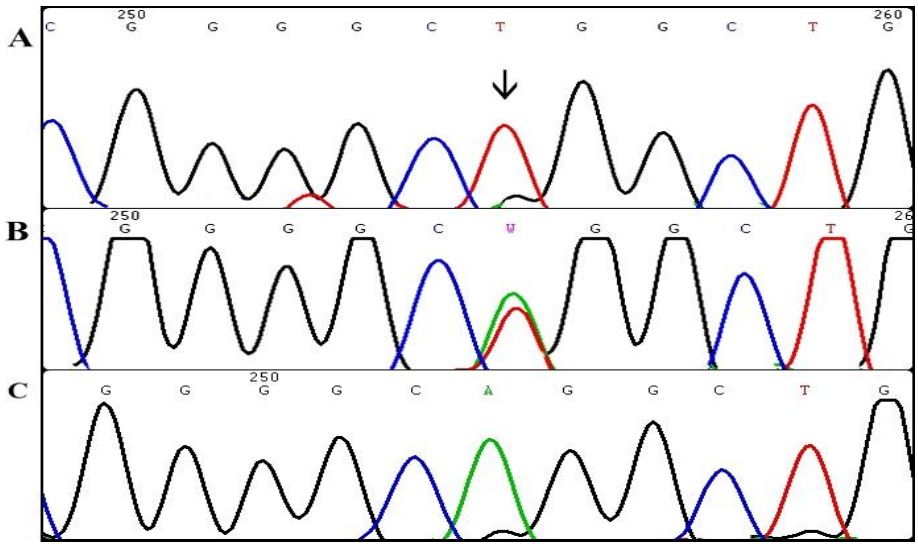

Figure 4. Chromatogram results of novel *MYO15A* variants in an Iranian family
A: normal alleles. B: A normal subject with hetrozygousc.6442T>A allele. C: A patient with homozygous c.6442T>A allele

Discussion

In the present study, we analyzed 30 families with ARNSHL and found one family (~3%) to be linked to DFNB3 by genetic linkage analysis (GLA). After DNA sequencing of the *MYO15A* gene, a novel variant (c.6442T>A, p.Trp2148Arg) was identified in the first MyTH4 domain. *MYO15A* with 66 coding exons encodes an unconventional myosin (myosin XV) that is expressed in the cochlea (19). This protein has important roles in the differentiation and elongation of the inner ear hair cell stereocilia , and it is also necessary for actin organization in hair cells (28). Mutations that cause hearing loss were first identified at the DFNB3 locus, in residents of a village in Indonesia. It was estimated that mutation frequencies were 9% among the inbred population. Since then, many mutations have been reported from different countries such as Pakistan, India, Turkey, Indonesia, and Brazil, interestingly, all of which belong to non-Caucasian populations (13, 14). Recently two compound heterozygote variants have been reported in Korean and Chinese families (15, 29). In addition,a novel homozygous mutation (c.9316dupC) in second MyTh4 of the MYO15A gene was identified by exome sequencing and Sanger sequencing in a Chinese family with ARNSHL (30). The novel mutation, found in this study, is located in the first domain of myosin tail homology4 (MTH4). To date, 7 mutations have been identified in this domain that are missense mutations. The MyTH4 domain of myosin has some roles in microtubule binding, as well as in actin binding at the plasma membrane (15, 31). Some studies suggest that MyTH4/FERM domain in MYOXVA is essential for its localization to stereocilia tips, although, their specific function is not been clearly known (18, 32, 33). MyTh4 domain contains a high percentage of conserved amino acids relative to other domains of the protein(33). Therefore, mutations in this domain can have a significant effect on proper function and structure of the protein. The c.6442T>A mutation leads to substitution of tryptophan for arginine amino acid at codon 2148. Tryptophan is a highly hydrophobic amino acid with aromatic structure, while arginine is a hydrophilic and positive charged amino acid. This amino acid substitution interferes with protein/microtubules interaction and actin and consequently disrupts the normal auditory function. MyTH4 domain interacts with PDZ domain of whirlin protein which is a cytoskeletal scaffold protein and is essential for normal auditory function. The co-localization of MYOXVA and whirlin proteins is essential for the assembly of actin microfilaments at stereocilia tips. In addition to the change in protein structure, this mutation interferes with the interaction between these two proteins and prevents the formation of actin microfilaments that is required for normal hearing. Different studies on Iranian populations show that mutations in *GJB2* and SLC26A4 are the first and second hearing loss causing mutations, respectively(34). To date, up to 10 mutations in the *MYO15A* gene have been identified in the Iranian population. Thus, according to the current studies the DFNB3 locus in hearing loss might rank third after *GJB2* and *SLC26A4*.

Conclusion

We have identified a novel mutation in the *MYO15A* gene in an Iranian family. This mutation affects the first MyTH4 domain of the protein. This region is highly conserved and should be investigated in future studies.

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